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Laboratory Guidebook
Notice of Change

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Title: Detection and Isolation of non-O157 Shiga-toxin Producing *Escherichia coli* Strains (STEC) from Meat Products

Effective Date: 10/01/10

Description and purpose of change(s):

This chapter describes procedures to detect and isolate non-O157 STEC serogroups including O26, O45, O103, O111, O121 and O145. The method utilizes a multiplex Real Time-PCR detection assay followed by cultural isolation. The multiplex Real Time-PCR assay detects the presence of the *stx* gene and *eae* gene. Further, multiplex PCR assays are used to identify the *wzx* gene, which encodes the O-antigen flippase within the O-antigen gene cluster. The *wzx* gene shows variability between serogroups and will hence be used as a diagnostic marker for the specific serogroup. Cultural isolation of non-O157 STEC proceeds using immunomagnetic separation beads (IMS) coated with serogroup-specific antibodies followed by plating onto Rainbow Agar. Typical colonies from Rainbow Agar are confirmed using the multiplex PCR assays and biochemical identification.

The methods described in this guidebook are for use by the FSIS laboratories. FSIS does not specifically endorse any of the mentioned test products and acknowledges that equivalent products may be available for laboratory use.

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5B.1 Introduction

Shiga-toxin producing *Escherichia coli* strains (STEC) of various serotypes have become an increasing public health concern since *E. coli* O157:H7 was first identified in 1982. STEC has been implicated in numerous outbreaks including development of hemolytic uremic syndrome (HUS) in some patients. Although *E. coli* O157:H7 has been most commonly identified as the cause of STEC infection, isolation of non-O157 STEC strains from clinical cases, outbreaks and environmental sources has been increasing (Posse *et al.*, 2008). A study at the Centers for Disease Control and Prevention showed that from 1983-2002 approximately 70% of non-O157 STEC infections were caused by one of six major serotypes, including O26, O45, O103, O111, O121 and O145 (Brooks *et al.*, 2005). Non-O157 STEC virulence factors include production of the shiga-like toxins 1 and/or 2 (Stx1, Stx2) and intimin (*eae)* genes.

Cattle and other ruminant animals appear to be the main reservoir of non-O157 STEC, as well as the O157 serotype (Arthur *et al.*, 2002). With carriage rates of non-O157 in cattle

being a public health concern, a method was devised to detect and isolate the six major non-O157 serotypes (O26, O45, O103, O111, O121 and O145) in ground beef and beef trim. This method, developed by USDA-Agricultural Research Services Eastern Regional Research Center (USDA-ARS-ERRC) in Wyndmoor, Pennsylvania, utilizes a multiplex Real Time-PCR detection assay followed by cultural isolation. The multiplex Real Time-PCR assay detects the presence of the *stx* gene and *eae* gene. Note that while this assay detects shiga toxin, it does not differentiate between stx1 and stx2. Further, multiplex PCR assays are used to identify the *wzx* gene, which encodes the O-antigen flippase within the O-antigen gene cluster. The *wzx* gene shows variability between serogroups and will hence be used as a diagnostic marker for the specific serogroup. Cultural isolation of non-O157 STEC proceeds using immunomagnetic separation beads (IMS) coated with serogroup-specific antibodies followed by plating onto Rainbow Agar. Typical colonies isolated from Rainbow Agar are confirmed using the multiplex PCR assays and biochemical identification.

5B.2 Safety Precautions

Similar to *E. coli* O157:H7, non-O157 STEC serotypes are human pathogens with a low infectious dose. The use of gloves and eye protection is mandatory for all post enrichment viable culture work. Work surfaces must be disinfected prior to and immediately after use. Laboratory personnel must abide by CDC guidelines for manipulating Biosafety Class II pathogens. A Class II laminar flow biosafety cabinet is recommended for activities with potential for producing aerosols of pathogens. All available Material Safety Data Sheets (MSDS) should be obtained from the manufacturer for the media, chemicals, reagents and microorganisms used in the analysis. The personnel who will handle the materials should read all MSDS sheets.

5B.3 Equipment, Reagents and Media

5B.3.1 Equipment and Materials

a. Balance, sensitivity to 0.1 g
b. Incubators, static 42 ± 1°C and 35 ± 2°C
c. Micropipettors for culture plating to deliver volumes ranging from 15-1000 µl with sterile disposable filtered tips
d. Micropipettors for RT-PCR to deliver volumes ranging from 0.25 µl to 1,000.00 µl with sterile, filtered tips
e. Cepheid® SmartCycler II

Cepheid® SmartCycler II PCR tubes with 25 µl volume, Cepheid Catalog Number 900-0022, or equivalent

f. VITEK® system, VITEK2® system, or equivalent biochemical identification system
g. Centrifuge capable of holding SmartCycler PCR tubes
h. 95-100° C water bath for DNA preparation step
i. Heating block or thermocycler for DNA preparation step
j. Vortex
k. Large tabletop centrifuge capable of speeds up to 16,000 *x*g
l. Disposable, sterile pipettes for volumes 1.0 ml and for 5.0 ml.
m. Sterile, inoculating loops, "hockey sticks" or spreaders, and needles
n. LabQuake® Agitator (or equivalent) with clips to hold microcentrifuge tubes
o. Sterile, disposable 12 x 75 mm polypropylene, or polystyrene, tubes (e.g. Fisher # 14-956-1B, or equivalent)
p. Sterile 1.5 ml microcentrifuge tubes
q. Sterile 50 ml conical tubes (e.g. Falcon® # 2070, or equivalent)
r. Sterile 40 µm Cell Strainer (Falcon® # 2340, or equivalent)
s. MACS® Large Cell Separation Columns (Miltenyi Biotec # 422-02, or equivalent)
t. OctoMACS® Separation Magnet (Miltenyi Biotec # 421-09, or equivalent)
u. Multistand to support OctoMACS® Separation Magnet (Miltenyi Biotec # 423-03, or equivalent)
v. Tray, autoclavable, approximately 130 mm x 83 mm (e.g. VWR # 62663-222, or equivalent) for use with the OctoMACS®
w. Stomacher™ 3500 with or without mesh (Tekmar Co., Cincinnati, Ohio), Bagpage® + 3500 (Interscience Lab. Inc., Boston MA) or equivalent bag mixer and bags

5B.3.2 Media and Reagents

a. Modified TSB broth with novobiocin (20 mg/L) plus casaminoacids (mTSB+n) Oxoid (or equivalent)
b. Rainbow® Agar O157 (Biolog Inc., Hayward California, 94545) containing 10 mg/L novobiocin plus 0.8 mg/L potassium tellurite, or equivalent selective medium

c. Tryptic soy agar with 5% sheep blood
d. E Buffer, approximately 7 ml per sample (Buffered Peptone Water, Bovine Albumin Sigma # A7906-500G, or equivalent and Tween-20®, or equivalent)
e. Disinfectant (Lysol® I. C., 2.0%, or equivalent)
f. Invitrogen Dynabeads® anti-O26 antibody-coated paramagnetic beads (Invitrogen catalog number 710.13, Invitrogen), anti-O103 antibody-coated paramagnetic beads (Invitrogen catalog number 710.11), anti-O111 antibody-coated paramagnetic beads (Invitrogen catalog number 710.09), anti-O145 antibody-coated paramagnetic beads (Invitrogen catalog number 710.07) or equivalent
g. Non-commercially available anti-O45 antibody-coated paramagnetic beads and anti-O121 antibody-coated paramagnetic beads. An equivalent commercially available product may be used as an alternative once available.
h. PrepMan® Ultra Sample Preparation Reagent(Applied Biosystems, Catalog number 4322547), or equivalent for DNA preparation from Gram-negative bacteria
i. Molecular grade water for PCR reactions
j. Omnimix™ PCR reagent beads (1.5U TaKaRa hot start *Taq* polymerase, 200 µM dNTP, 4 mM $MgCl_2$, and 25mM HEPES, pH 8.0, per 25 µl reaction), Cepheid catalog number OMNI1-100N-050
k. Biochemical test kit and system, GNI and GNI Plus cards (VITEK® system, bioMerieux Vitek, Inc., 595 Anglum Drive, Hazelwood, MO 63042-2395), GN cards (VITEK® 2 system), or equivalent
l. Primers and probes will be provided as a primer/probe mixture by FSIS-OSEL.

5B.4 Quality Control

5B.4.1 General

a. Rainbow Agar plates have a shelf life of two weeks.
b. All media, plates and buffers must be warmed to 18-35° C prior to use.
c. All non-commercially available critical supplies lacking a Certificate of Analysis must have quality control lot acceptance performed for each lot and shipment before use.

d. Stock primer concentrations should be reconstituted to 200.0 µM and stored ≤ -20° C. Working primers concentrations will be diluted to 20.0 µM (1:10 of stock) and stored ≤ -20° C.

e. Stock probe concentrations should be reconstituted to 20.0 µM and stored ≤ -20° C. Working primers concentrations will be diluted to 2.0 µM (1:10 of stock) and stored ≤ -20° C. Both stock and working probes should be stored in the dark.

5B.4.2 Sample Enrichment Controls

Sample batches shall include a positive growth control, which will be the bioluminescent *E. coli* O157:H7 (FSIS culture# 465-97) and an uninoculated media control.

5B.4.3 DNA Extraction Control Preparation

Laboratories will also use bioluminescent *E. coli* O157:H7 465-97 as a postive DNA extraction control. Note: The bioluminescent *E. coli* O157:H7 is negative for the stx target but is positive for the *eae* target.

With each sample batch, extract DNA from an aliquot using the same extraction method as used to process the sample batch. This will serve as a positive DNA extraction control.

5B.4.4 PCR Controls

a. *stx/eae* screen PCR
- DNA template from bioluminescent *E. coli* O157:H7 (DNA extraction control)
- DNA template from toxigenic *E. coli* O157:H7 (provided to FSIS laboratories by OSEL)
- No Template Control (NTC)

b. *wzx* screen PCR
- DNA template from reference strains (provided to FSIS laboratories by OSEL).
- DNA from serogroups assayed for in the same multiplex PCR have been paired and will be used as one control for the *wzx* multiplex PCR assay.
- NTC

c. *stx/eae* confirmatory PCR

- DNA template from toxigenic *E. coli* O157:H7 (provided to FSIS laboratories by OSEL)
- NTC

d. *wzx* screen PCR

- DNA template from reference strain(s) assaying for (provided to FSIS laboratories by OSEL). DNA from serogroups assayed for in the same multiplex PCR have been paired and will be used as one control for the *wzx* mulitplex PCR assay.
- NTC

5B.4.5 IMS Plating Controls

Streak the serogroup(s) of interest (based on PCR *wzx* results) onto Rainbow Agar and incubate along with the samples that have been subject to IMS isolation.

5B.5 Sample Preparation and Primary Enrichment

Note: Disinfect the sample package prior to opening.

a. Place the 325±32.5g test portion per submitted sample into the provided sterile bag with filter. Ensure that the entire test portion is on the same side of the filter.
b. Dilute the test portion in 975 ± 19.5 ml (1:4 dilution) of modified TSB + casamino acids and novobiocin (mTSB).
c. Incubate the test portion and the enrichment media at 42±1 °C for 22-24 hours. Each group of samples should include a positive control enrichment (bioluminescent *E.* coli O157:H7 strain, FSIS culture # EC 465-97) and an uninoculated medium control.

5B.6 Screening Procedure using Real-Time PCR

5B.6.1 DNA Extraction from Overnight Enrichments

a. Remove a 1.5 ml aliquot of each sample following overnight enrichment and transfer to labeled, sterile microcentrifuge tubes.
b. Centrifuge the tubes with a setting of 2,000 *x*g for 1 minute to pellet any larger meat particles.
c. Transfer the supernatant to another sterile microcentrifuge tube. Centrifuge the tubes at a setting of 16,000 *x*g for 3 minutes. This step will pellet the bacteria present in the enrichment.

d. Discard the supernatant while leaving the pellet at the bottom of the tube. Add 200 µl of PrepMan® Ultra Reagent to each pellet and vortex vigorously until the solution is homogenous.
e. Heat the solution at 95-100 °C for 10±1 minutes. Remove the tubes from the heating block/water bath/thermocycler.
f. Centrifuge the tubes at a setting of 16,000 *x*g for 3 minutes at room temperature or refrigerated. Transfer the liquid supernatant to new, sterile microcentrifuge tubes. Do not disturb the pellet.
g. Hold all extracted DNA on ice or at 2-8 °C, if testing that day. Freeze DNA extractions at ≤ -20 °C for long-term storage.

5B.6.2 Real-Time PCR Procedure

Following DNA extraction from overnight enrichments, Real-Time PCR will be used as a screen for the presence of *stx* toxin genes and the *eae* intimin gene. Samples with positive hits on the initial Real-Time PCR screen will be subjected to another Real-Time PCR that will yield the serogroup (O26, O45, O103, O111, O121 or O145) based on amplification and detection of the *wzx* (O-antigen flippase) gene.

a. For the initial *stx* and *eae* PCR screen, prepare master mix as shown in Table 1. Pre-prepared primers and probes master mix will be provided to FSIS laboratories by OSEL. Laboratories will need to add the indicated amount of water and indicated number of OmniMix PCR beads. **Note:** One Omnimix™ PCR reagent bead is used for 2 PCR reactions. If receiving lyophilized primers and probes, dilute primers to a working concentration of 20.0 µM and probes to a working concentration of 2.0 µM for storage at ≤ -20 C.

Table 1. Initial *stx* and *eae* PCR Screen Mastermix (per 2 reactions)

One Omnimix™ PCR reagent bead

	Initial Concentration	Final Concentration	Vol. per 2 reactions
Primer 16SRna-F	20.0 µM	O.20 µM	0.5 µl
Primer 16SRna-R	20.0 µM	O.20 µM	0.5 µl
Primer Eae223-F	20.0 µM	O.20 µM	0.5 µl
Primer Eae223-R	20.0 µM	O.20 µM	0.5 µl
Primer Stx1-150-F	20.0 µM	O.25 µM	0.625 µl
Primer Stx1-150-R	20.0 µM	O.25 µM	0.625 µl
Primer Stx2-200-F	20.0 µM	O.25 µM	0.625 µl
Primer Stx2-200-R	20.0 µM	O.25 µM	0.625 µl
Probe Eae188-P	2.0 µM	O.125 µM	3.125 µl
Probe Stx1-150-P	2.0 µM	O.1875 µM	4.688 µl
Probe Stx2-200-P	2.0 µM	O.1875 µM	4.688 µl
Probe 16SRna-P	2.0 µM	O.125 µM	3.125 µl
Molecular Grade Water	N/A	N/A	24.875 µl

Total Volume = 45.0 µl

b. For each PCR reaction, aliquot 22.5 µl of mastermix into a sterile, SmartCycler II PCR tube and keep on PCR cold block.
c. Add 2.5 µl of DNA template from the DNA extraction step to the appropriate tube. Gently centrifuge the tubes once ready to load on the SmartCycler II platform.
d. Enter a program titled "*stx* and *eae* non-O157 PCR" into the SmartCycler using the parameters described in Table 2. The dye set to be used in the PCR assays is FCTC25 (FAM, Cy3, TxRed and Cy5).
e. Load the tubes into the SmartCycler II and begin the assay. See Table 3 for PCR result interpretation.

Table 2. Non-O157 Real-Time PCR Assay Parameters

stx and *eae*		
1 Cycle	95°C	120 seconds
45 Cycles	95°C	10 seconds
	60°C	45 seconds with Optics On

O26 and O111		
1 Cycle	95°C	120 seconds
45 Cycles	95°C	10 seconds
	62°C	30 seconds with Optics On
	72°C	30 seconds

O45 and O121		
1 Cycle	94°C	120 seconds
45 Cycles	94°C	10 seconds
	57°C	20 seconds with Optics On
	72°C	20 seconds

O103 and O145		
1 Cycle	95°C	120 seconds
45 Cycles	95°C	10 seconds
	58°C	20 seconds with Optics On
	72°C	20 seconds

Table 3. Result interpretation for the *stx* and *eae* PCR assays.

	stx PCR (TxRed) Negative	*stx* PCR (TxRed) Positive	16s RNA PCR (Cy5) Negative
eae PCR (FAM) Negative	* Negative * STOP Report Result	* Negative * STOP Report Result	* Indeterminate * Repeat *stx* and *eae* PCR
eae PCR (FAM) Positive	* Negative * STOP Report Result	Continue with *wzx* PCR for Serogroup	
16s RNA PCR (Cy5) Negative	* Indeterminate * Repeat *stx* and *eae* PCR		

f. At the end of the run, view the results table to determine if samples are positive for *eae* and *stx* targets (FAM channel and TexasRed channel, respectively). A gene target that crosses over the threshold value is considered positive. The 16S rRNA internal control labeled with Cy5 should be positive for every sample except the No-Template Control.

DNA extractions from any samples positive for both *stx* and *eae* targets will be further analyzed by PCR amplification of the *wzx* gene targets specific for each serogroup. This will be accomplished through three multiplex Real-Time PCR reactions.

g. For the *wzx* serogroup-specific PCR screen, prepare master mix as shown in Table 4. The pre-*pre*pared primers and probes master mix will be provided by OSEL. Laboratories will need to add the indicated amount of water and indicated number of Omnimix PCR beads. **Note**: One Omnimix™ PCR reagent bead is used for 2 PCR reactions. If receiving lyophilized primers and probes, dilute primers to a working concentration of 20.0 µM and probes to a working concentration of 2.0 µM for storage at ≤ -20 C.

Table 4. *wzx* PCR Screen Mastermix (per 2 reactions)

I. *Multiplex PCR assay for O26 and O111.*

One Omnimix™ PCR reagent bead

	Initial Concentration	Final Concentration	Vol. per 2 reactions
Primer 16SRna-F	20.0 µM	O.20 µM	0.5 µl
Primer 16SRna-R	20.0 µM	O.20 µM	0.5 µl
Primer Wzx158-O26-F	20.0 µM	O.25 µM	0.625 µl
Primer Wzx158-O26-R	20.0 µM	O.25 µM	0.625 µl
Wzx237-O111-F	20.0 µM	O.25 µM	0.625 µl
Wzx237-O111-R	20.0 µM	O.25 µM	0.625 µl
Probe Wzx158-O26-P	2.0 µM	O.10 µM	2.5 µl
Probe Wzx237-O111-P	2.0 µM	O.20 µM	5.0 µl
Probe 16SRna-P	2.0 µM	O.125 µM	3.125 µl
Molecular Grade Water	N/A	N/A	30.875 µl

Total Volume = 45.0 µl

II. *Multiplex PCR assay for O45 and O121.*

One Omnimix™ PCR reagent bead

	Initial Concentration	Final Concentration	Vol. per 2 reactions
Primer 16SRna-F	20.0 µM	O.20 µM	0.5 µl
Primer 16SRna-R	20.0 µM	O.20 µM	0.5 µl
Primer Wzx72-O45-F	20.0 µM	O.25 µM	0.625 µl
Primer Wzx72-O45-R	20.0 µM	O.25 µM	0.625 µl
Primer Wzx189-O121-F	20.0 µM	O.25 µM	0.625 µl
Primer Wzx189-O121-R	20.0 µM	O.25 µM	0.625 µl
Probe Wzx72-O45-P	2.0 µM	O.1875 µM	4.688 µl
Probe Wzx189-O121-P	2.0 µM	O.1875µM	4.688 µl
Probe 16SRna-P	2.0 µM	O.125 µM	3.125 µl
Molecular Grade Water	N/A	N/A	29.0 µl

Total Volume = 45.0 µl

III. *Multiplex PCR assay for O103 and O145*

One Omnimix™ PCR reagent bead

	Initial Concentration	Final Concentration	Vol. per 2 reactions
Primer 16SRna-F	20.0 µM	O.20 µM	0.5 µl
Primer 16SRna-R	20.0 µM	O.20 µM	0.5 µl
Primer Wzx191-O103-F	20.0 µM	O.25 µM	0.625 µl
Primer Wzx191-O103-R	20.0 µM	O.25 µM	0.625 µl
Primer Wzx222-O145-F	20.0 µM	O.25 µM	0.625 µl
Primer Wzx222-O145-R	20.0 µM	O.25 µM	0.625 µl
Probe Wzx191-O103-P	2.0 µM	O.20 µM	5.0 µl
Probe Wzx72-O145-P	2.0 µM	O.10 µM	2.5 µl
Probe 16SRna-P	2.0 µM	O.125 µM	3.125 µl
Molecular Grade Water	N/A	N/A	30.875 µl

Total Volume = 45.0 µl

h. For each PCR reaction, aliquot 22.5 µl of mastermix into a sterile, SmartCycler II PCR tube and keep on PCR cold block.

i. Add 2.5 µl of DNA template from the DNA extraction step to the appropriate tube. Gently centrifuge the tubes once ready to load on the SmartCycler II platform.
j. Enter programs titled "O26 and O111 non-O157 PCR screen", "O45 and O121 non-O157 PCR screen" and "O103 and O145 non-O157 PCR screen" into the SmartCycler using the parameters described in Table 2.
k. At the end of the run, view the results table to determine if samples are positive for the serogroup-specific *wzx* targets. The dye channels with their respective targets are as follows:
FAM = Serogroup O26, Serogroup O45 and Serogroup O145
TxRed = Serogroup O103, Serogroup O111 and Serogroup O121
Cy5 = Internal Control (16S rRNA)

A gene target that crosses over the threshold value is considered positive. The 16S rRNA internal control labeled with Cy5 should be positive for every sample except the No-Template Control.

5B.7 Isolation Procedure

PCR screen results will be used to determine the serogroup potentially present in the sample. In the isolation procedure, IMS beads will be used for the specific serogroup identified by the *wzx* PCR reaction (i.e. anti-O26 will be used for samples with screen results positive for O26, anti-O45 for O45 PCR positive reactions, anti-O103 for O103 PCR positive reactions or anti-O121 for O121 PCR positive reactions, anti-O111 for O111 PCR positive reactions and/or anti-O145 for O145 PCR positive reactions).

5B.7.1 Immunomagnetic Separation and Culture Plating

a. **Sample preparation from overnight enrichment:** For each serogroup that the sample is positive, transfer approximately 2-5 ml from overnight enrichment through 40 µm Cell Strainer into 50 ml conical centrifuge tubes.

b. **Binding of paramagnetic antibody beads to specific serogroup:** Transfer 1 ml of filtrate to a sterile, labeled microcentrifuge tube. To each milliliter of filtrate, add 5 µl of appropriate immunomagnetic capture beads (O26, O45, O103, O111, O121 or O145), as determined by the O-group (*wzx*) PCR screen results.

c. Place the microcentrifuge tubes containing enrichments and capture beads on LabQuake® Agitator (or equivalent) and rotate tubes for 10 minutes at room temperature.

d. For each sample, place one MACS® Large Cell Separation Columns onto the OctoMACS® Separation Magnet. Fill the tray below the separation magnet with disinfectant. Prime each separation column with 1000 µl of E-buffer and allow the liquid to pass completely through before adding sample.

e. **Binding of beads to magnetic columns:** Once the liquid has passed through the column, add the 1.0 ml of enrichment plus IMS beads to each appropriately labeled column and allow liquid to completely pass through.

f. **Wash steps (4X):** Add 1000 µl of E-buffer to each column allowing the liquid to pass completely through. Repeat this step 4 times.

g. **Elution step:** After the last wash has drained, remove the column from the OctoMACS® Magnet and insert the tip into an empty labeled 12 x 75 mm tube. Apply 1000 µl of E Buffer to the column, and using the plunger supplied with the column, immediately flush out the beads into the tube. Use a smooth, steady motion to avoid splattering. Cap the tubes. Repeat this for each column.

h. Make a 1:10 dilution of each treated bead suspension by adding 0.1 ml of the bead suspension to a 12 x 75 mm labeled tube containing 0.9 ml E Buffer.

i. Vortex briefly to maintain beads in suspension and plate 0.1 ml from each tube (undiluted and diluted) onto a labeled Rainbow® Agar plate. Use a hockey stick or spreader to spread plate the beads, being careful not to spread the beads against the edge of the plate.

j. Vortex the tubes containing undiluted beads (from step g) and transfer to a labeled microfuge tube and centrifuge at least one minute using a bench-top microcentrifuge to concentrate the beads. Withdraw and discard the supernatant without disturbing the beads. Add 0.1 ml of E Buffer to the beads, resuspend the beads and transfer the beads to a labeled Rainbow® Agar plate. Spread plate the beads as described in step i.

k. As soon as there is no visible moisture on the agar surface, invert plates and incubate for 20-24 h at 35 ± 2°C.

5B.8 Identification and Confirmation

Following 20-24 h incubation of plating media, plates will be examined for colonies exhibiting phenotypes typical of the six non-O157 STEC. Table 4 lists the typical phenotypes of each serogroup when grown on Rainbow Agar. Colonies exhibiting the correct phenotype will be confirmed with Real-Time PCR following heat preparation of the DNA. The confirmatory PCR will include the *stx* and *eae* multiplex PCR and the serogroup (*wzx*) specific multiplex PCR assay. Select up to 15 typical colonies per sample for confirmatory PCR and biochemical identification (VITEK). For the first 5 colony picks, plate onto Tryptic soy agar with 5% sheep blood (SBA). An additional 10 colony picks from each sample should be spotted to a grid, formatted non-selective agar (SBA, BHI or TSA). If at least one of the five initial picks does not confirm by both PCR and VITEK, streak another five picks from the grid plate onto SBA and repeat the confirmatory PCR. If none of the next five picks confirm by PCR and VITEK, streak the remaining five picks and repeat the PCR. PCR positive colonies should be restreaked onto SBA for VITEK biochemical confirmation.

Note: Each PCR assay should include a NTC negative control and a positive control for each gene target. Refer to Quality Control section for details on expected controls.

a. From Rainbow Agar, streak 5 colonies for isolation onto SBA agar and incubate 18-24 hours at 35-37°C. Also, spot an additional 10 picks onto a grid, formatted non-selective agar (SBA, BHI or TSA) and incubate 18-24 hours at 35-37°C. For colonies to be confirmed using PCR, resuspend a colony from the SBA plate in 50 µl of Molecular Grade Water and heat the preparation for 10 minutes at 95-100 °C.

b. From heated 50 µl suspension, perform the *stx* and *eae* multiplex PCR assay as previously described in Section 5B 6.2 using 2.5 µl of DNA template in the reaction. Also, perform the PCR multiplex assay described in Section 5B 6.2 specific for the serogroup of interest.

c. If none of the initial picks are PCR positive, repeat 5B.8 steps a-c for remaining picks plated on non-selective media. If the DNA template preparation from the colony is PCR positive, streak the PCR positive colony onto SBA and incubate 18-24 h at 35-37 °C.

d. From the SBA plate, confirm that the isolate is *E. coli* through biochemical identification (VITEK or equivalent biochemical identification test).

Table 4. Typical phenotypes of non-O157 STEC colonies on Rainbow Agar

E. coli Strain	Colony color on Rainbow Agar
O26	Purple
O45	Magenta, mauve
O103	Blue-violet
O111	Gray-black
O121	Pink with dark pink center
O145	Violet or light purple

5B.9 Selected References

Possé B, De Zutter L, Heyndrickx M, Herman L. (2008) Novel differential and confirmation plating media for Shiga toxin-producing Escherichia coli serotypes O26, O103, O111, O145 and sorbitol-positive and -negative O157. FEMS Microbiol Lett. 282(1):124-31.

Brooks JT, Sowers EG, Wells JG, Greene KD, Griffin PM, Hoekstra RM, Strockbine NA. (2005) Non-O157 Shiga toxin-producing Escherichia coli infections in the United States, 1983-2002. J Infect Dis. 192(8):1422-9.

Arthur TM, Barkocy-Gallagher GA, Rivera-Betancourt M, Koohmaraie M. (2002) Prevalence and characterization of non-O157 Shiga toxin-producing Escherichia coli on carcasses in commercial beef cattle processing plants. Appl Environ Microbiol. 68(10):4847-52.